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1 Comparison of *Listeria monocytogenes* exoproteomes from
 2 biofilm and planktonic state: Lmo2504 a protein associated with biofilms

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17 **Running title:** Biofilm and planktonic exoproteomes of *L. monocytogenes*

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27 **Abstract**

28 The foodborne pathogen *Listeria monocytogenes* is the causative agent of the severe
29 human and animal disease listeriosis. The persistence of this bacterium in food processing
30 environments is mainly attributed to its ability to form biofilms. The search for proteins
31 associated with biofilm formation is an issue of great interest, with most studies targeting
32 the whole bacterial proteome. Nevertheless, exoproteins constitute an important class of
33 molecules participating in various physiological processes such as cell signaling,
34 pathogenesis and matrix remodeling. The aim of this work was to quantify differences in
35 protein abundance between exoproteomes from biofilm and from planktonic state. For this,
36 two field strains previously evaluated as good biofilm producers (3119 and J311) were
37 used, and a procedure for the recovery of biofilm exoproteins was optimized. Proteins
38 were resolved by 2D-DIGE and identified by ESI-MS/MS. One of the proteins identified in
39 higher abundance in the biofilm exoproteomes of both strains was the putative cell wall
40 binding protein Lmo2504. A deletion mutant strain on this gene was produced
41 (3119 Δ Lmo2504) and its biofilm forming ability compared to the wild type using the crystal
42 violet and the ruthenium red assays, as well as scanning electron microscopy. The results
43 confirmed the involvement of Lmo2504 in biofilm formation, as strain 3119 Δ Lmo2504
44 showed significantly ($p < 0.05$) lower biofilm forming ability, compared to the wild type. The
45 identification of additional exoproteins associated with biofilm formation may lead to new
46 strategies for controlling this pathogen in food processing facilities.

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50 **Keywords:** *Listeria monocytogenes*, exoproteome, biofilm, planktonic, Lmo2504 (putative
51 cell wall binding protein).

52 1. Introduction

53 *Listeria monocytogenes* is a foodborne pathogen able to persist in food processing environments. It is
54 the causative agent of the severe human and animal disease, invasive listeriosis, whose features
55 frequently include meningitis or sepsis. Listeriosis has a mortality rate of 23.7 % (1) and is especially
56 severe in the elderly and in immunocompromised persons. In the case of pregnant women it may
57 lead to premature birth, abortion or stillbirth.

58 The ability of these bacteria to form biofilms is often associated with their ability to survive adverse
59 conditions in food processing environments. Its biofilm forming ability has been evaluated by several
60 methods more or less correlated to the conditions found in the food industry, namely the type of
61 surface material (2). In addition to microbial cells, the biofilm matrix is composed by
62 exopolysaccharides, lipids, glycolipids, DNA and proteins (3). The evaluation of biofilm forming ability
63 may be performed by several methods that target different biofilm components. Methods such as the
64 crystal violet assay (4) are directed towards the viable cells within the biofilm, and methods such as
65 the ruthenium red assay (4) target the biofilm matrix exopolysaccharides. A more reliable comparison
66 between strains is obtained when agreement between such different approaches is verified.

67 The role of proteins within the biofilm matrix has also been demonstrated by the decrease in adhesion
68 which occurs after treatment of *L. monocytogenes* cells with proteases (5).

69 The cells within biofilms are characterized by gene expression patterns different from their planktonic
70 counterparts. Bacterial transcriptomic analysis does not always correlate with detected proteins and
71 their functional activity. The proteomic approach allows a glimpse into the presence of functional
72 molecules (6).

73 The aim of this work was to evaluate differences in exoproteins from biofilm cells *versus* its planktonic
74 counterpart by using two-dimensional difference gel electrophoresis (2D DIGE) and electrospray
75 tandem mass spectrometry (ESI-MS/MS) identification. We have developed a suitable method to
76 obtain an appropriate amount of biofilm exoproteins for analysis and to minimize the contamination

77 with proteins from planktonic cells. Protein Lmo2504, putative cell wall binding, was one of the most
78 abundant proteins in the biofilm exoproteomes. A deletion mutant on the gene coding for this protein
79 was constructed and its biofilm forming ability compared with the wild type. This allowed to
80 experimentally confirm, for the first time, the involvement of this protein in biofilm formation.

81

82 **2. Materials and methods**

83 **2.1. Strains**

84 Two field strains of *L. monocytogenes*, previously identified as good biofilm producers (2), were
85 selected for this study: strain J311 (serovar 4b) was isolated from raw chicken (7) and strain 3119
86 (serovar 1/2b) was isolated from cheese (8). To construct a mutant of strain 3119, *Escherichia coli*
87 strain DH5 α carrying pAUL-A (9) was used. For mutant construction, *E. coli* strain was grown at 37 °C
88 with shaking in LB medium or on LA plates and when required, erythromycin was added to a final
89 concentration of 250 $\mu\text{g ml}^{-1}$. *L. monocytogenes* strains were grown at 37 °C with shaking in BHI
90 broth or on BHI plates and when required, erythromycin was added to a final concentration of 5 $\mu\text{g ml}^{-1}$.
91 ¹. Cultures were stored at -80 °C in Tryptic Soy Broth or LB with 15% (w/v) glycerol, until use.

92 **2.2. Growth of biofilm and planktonic cultures and protein secretion**

93 Cultures from -80 °C were struck onto TSA-YE (Oxoid, Hampshire, UK) and incubated overnight at 25
94 °C. For each isolate, 10 ml of Modified Welshimer's broth (MWB) (10) was inoculated and incubated
95 24 hours at 25 °C to obtain pre-inocula.

96 For planktonic growth, 1 ml of each pre-inoculum was used to inoculate 30 ml of MWB and incubated
97 for 24 h at 25 °C at 150 rpm. The cultures were then centrifuged at 3000 g for 20 minutes and the
98 supernatant fluid discarded. Cells were resuspended in 30 ml of fresh MWB and centrifuged again to

99 discard the supernatant fluid. Thirty ml of fresh MWB was used to resuspend the pellet and incubation
100 proceeded for 6 hours at 25 °C for protein secretion.
101 Biofilms were grown on stainless steel coupons prepared as described by Lourenco et al.
102 (2). Disposable test tubes with 24 ml of MWB were inoculated with 1 ml of each pre-inoculum and
103 incubated for 24 h at 25 °C. After this period, 14 stainless steel coupons were immersed in the
104 cultures and incubated for 4 hours at 25 °C to allow attachment. After this contact period, unattached
105 cells were removed by replacing the media with 25 ml of fresh MWB. The biofilm was then allowed to
106 form by incubating 48 h at 25 °C followed by media renewal and subsequent incubation of another 48
107 hours. After biofilm formation, the biofilm was rinsed with 25 ml of fresh MWB to remove planktonic
108 cells. Twenty five ml of fresh MWB was placed in contact with the biofilm, for 6 hours at 25 °C, to
109 allow for protein secretion. Three biological replicates were obtained for each strain-condition
110 combination.

111 **2.3. Protein precipitation and quantification**

112 Proteins were precipitated from culture supernatant fluids as described by Cabrita et al. (11). Just
113 before use, protein samples were dissolved in 300 µl of buffer solution A (8 M urea, 2 M thiourea, 4 %
114 CHAPS, 20 mM Tris pH 8.5, 0.2% SDS). Two µl of Benzonase® nuclease (Novagen, Madison WI,
115 USA) and MgCl (final concentration of 5 mM) were then added. The protein concentration in the
116 samples was determined using the EZQ™ Protein Quantitation Kit according to manufacturer
117 instructions (Invitrogen, Ltd., USA).

118 **2.4. Two-dimensional Difference Gel Electrophoresis (2-D DIGE)**

119 A total of 35 µg of protein from each sample was labeled with a different CyDye according to the three
120 dye protocol for minimal labeling (GE Healthcare Inc., USA). Briefly, adding dye solution (400
121 pmol/µl), vortexing, centrifuging and incubating on ice, in the dark, for 30 min. The labeling reaction
122 was then stopped by adding 1 µl of 10 mM lysine and incubation on ice, in the dark, for 10 min. An

123 internal standard pool was created from all of the samples and 35 µg was also labeled for use in each
124 gel. Dye swapping between experimental samples was performed to control dye-specific artifacts. In
125 each gel, 120 µg of unlabeled protein was added. The total volume of the samples was normalized to
126 500 µl by adding Dithiothreitol (DTT) solution to a final concentration of 100 mM, IPG buffer pH 4-7
127 and 2 µl of Orange G dye (Fisher Scientific, Fair Lawn NJ, USA) and buffer solution A. The samples
128 were loaded on IPG strips (24 cm pH 4-7) (GE Healthcare Biosciences AB, Uppsala, Sweden) and
129 rehydrated overnight (16 -18 hours). An Ettan IPGphor 3 (GE Healthcare) was used for isoelectric
130 focusing. The proteins on IPG strips were then reduced and alkylated in NuPAGE® LDS Sample
131 Buffer (Invitrogen) with 100 mM DTT and then 2.5 % iodoacetamide. The strips were subsequently
132 loaded on 8.0 % to 16.0 % Tris-Glycine gradient, 1 mm thick gels (Jule Inc. Biotechnologies, Milford
133 CT, USA). A 25 -225 kDa ladder (GE Healthcare) was loaded in each gel. The electrophoresis of the
134 six gels was performed simultaneously in an Ettan DALTsix (GE Healthcare) at 12 °C with an initial
135 step of 80 V for one hour at 1 W/gel followed by an overnight run at 150 V and 2 W/gel.

136 **2.5. Gel analysis**

137 Gels were scanned with a Thyphoon Trio + scanner (GE Healthcare) with a pixel size of 100 µm
138 using the appropriate excitation lasers and emission filters depending on the dye according to
139 manufacturer (GE Healthcare). The gel images were analyzed using DeCyder™ image analysis
140 software, Biological Variation Analysis (BVA) module (GE Healthcare). Spots were selected based on
141 a fold difference higher than 1.5 and with a significance of $p < 0.05$ (Student's t test for paired
142 samples) excised using Investigator™ ProPic protein picking robot (Genomic Solutions Ltd, Ann
143 Arbor MI, USA) and combined. The samples were destained twice with 50 % (v/v) ACN, and 50 mM
144 ammonium bicarbonate (ABC) (Fisher Scientific) solution. The solution was then removed and dried
145 using a speed vacuum centrifuge (Labconco Corporation, Kansas City MO, USA).
146 The samples were rehydrated in 100 µl of 45 mM DDT at 55 °C for 45 min. The tubes were chilled to
147 room temperature, DTT quickly removed and replaced by 100 µl of 100 mM iodoacetamide (IAA)

148 followed by incubation in the dark at room temperature, for 45 minutes. IAA was removed and the
149 samples washed three times with 1:1 ACN:ABC solution. The gel pieces were then dried down in
150 speed vacuum for 15 minutes. Protein in-gel digestion was obtained using 10 µl of a 12.5 ng/µl
151 solution of porcine trypsin (Promega, Madison WI, USA) and covering it with 30 µl of ABC to keep the
152 gel pieces immersed throughout the digestion of 12 hours at 37 °C. Peptides were extracted by
153 removing the supernatant fluid to fresh tubes. Digestion tubes were washed with 200 µl of 80 % ACN, 0.1
154 % formic acid (FA) in order to increase the peptide recovery. The samples were speed dried.
155 For mass spectrometry the peptides were solubilized in 11 µl of a solution 30 % ACN, 1 M FA. The
156 samples were then sonicated for two minutes and vortexed. The samples were subsequently washed
157 using Zip Tip® Pipette tips C18 (Millipore, Billerica MA USA) with 0.1 % FA solution, and eluted from
158 the Zip tips using 5 µl of a 20 % isopropyl alcohol (IPA), 30 % ACN, and 0.1 % FA solution.
159 After prediction of the volume of protein present in each sample, the ones with higher concentration
160 were identified in ESI-MS/MS through direct infusion. The less concentrated samples were dried and
161 solubilized in 50% ACN, 0.1% FA for high performance liquid chromatography – electrospray tandem
162 mass spectrometry (HPLC-ESI-MS/MS).

163 **2.6. Peptide sequencing by ESI-MS/MS**

164 LC-MS/MS: The enzymatically digested samples were injected onto a C18 trap column (SGE
165 Incorporated, TX) and desalted for 5 min with a flow rate 3 µl/min of 0.1% v/v formic acid. The
166 samples were loaded onto an LC Packing® C18 Pep Map nanoflow HPLC column. The elution
167 gradient of the HPLC column started at 3 % solvent B, 97 % solvent C and finished at 50% solvent B,
168 40% solvent C for 20 min then brought back to initial conditions for protein identification. Solvent B
169 consisted of 0.1% v/v formic acid, 96.9% v/v ACN, and 3% v/v H₂O. Solvent C consisted of 0.1% v/v
170 formic acid, 3% v/v ACN, and 96.9% v/v H₂O.

171 A LTQ Orbitrap XL mass spectrometer (ThermoFisher Scientific, West Palm Beach, FL) was used for
172 LC-MS/MS analysis. The ion spray voltage was set to 2200 V. Full MS scans were acquired with a

173 resolution of 60,000 in the orbitrap from m/z 300–2000. The ten most intense ions were fragmented by
174 collision induced dissociation (CID). Dynamic exclusion was set to 60 seconds.

175 **2.7. Protein Search Algorithm**

176 MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.2.2). Mascot
177 was set up to search a *L. monocytogenes* database extracted from NCBI assuming digestion with
178 trypsin. Mascot was searched with a fragment ion mass tolerance of 0.8 Da and a parent ion
179 tolerance of 15 ppm. Iodoacetamide derivative of Cys, deamidation of Asn and Gln, oxidation of Met,
180 were specified in Mascot as variable modifications. Scaffold (version Scaffold-3.3.2, Proteome
181 Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications.
182 Peptide identifications were accepted if they could be established at greater than 95.0% probability as
183 specified by the Peptide Prophet algorithm (12). Protein identifications were accepted if they
184 established at greater than 99.0% probability and they contained, at least, two identified unique
185 peptides. Protein probabilities were assigned by the Protein Prophet algorithm (13).

186 **2.8. Construction of deletion mutant strain (*L. monocytogenes* 3119Δ*Imo2504*)**

187 Genome sequence of strain FSL J1-175 (from the same serovar 1/2b) provided by the BROAD
188 Institute (<http://www.broad.mit.edu>) was used for primer design to generate the isogenic in-frame
189 deletion mutant of strain 3119. Two flanking regions, upstream and downstream of the gene
190 homologous to *Imo2504* in strain EGDe, with approximately 700 basepair (bp) each, were generated
191 by PCR from strain 3119 genomic DNA, using primers P1 together with P2 and P3 together with P4
192 (Table 1), respectively. The PCR product obtained by using primers P1 and P4 and using as
193 templates the up and downstream regions digested with *Bam* HI and *Eco* RI, was ligated to the
194 temperature-sensitive suicide vector pAUL-A (9) previously linearized with the same enzymes. The
195 resulting plasmid (pFARRUQ) was electroporated using Gen Pulser® (Biorad) set at 400 Ω, 10 kV/cm,

196 25 μ F, into strain 3119, integrated and excised as described by Arnaud et al. (14) for homologous
197 recombination.

198 The resulting mutant strain (3119 Δ *mo2504*) was confirmed by PCR analysis using primers P5
199 together with P6 (flanking the gene), P7 together with P8 (intragenic region) and also P8 together with
200 P5 and P7 together with P6 (Table 1).

201

202 **2.9.Evaluation of biofilm forming ability: crystal violet, ruthenium red and scanning** 203 **electron microscopy (SEM)**

204 For SEM, biofilms of wild type and mutant strain 3119 Δ *mo2504* were grown on 1×1 cm stainless
205 steel coupons as described in section 2.2. After biofilm formation, the coupons were washed with
206 phosphate buffer, fixed with glutaraldehyde 2.5 % (v/v) for 3 hours and washed by overnight
207 immersion in phosphate buffer. Biofilms were then dehydrated in a 10 minutes steps gradient of 30-
208 50-70-90-100 % (v/v) ethanol. Each coupon was attached to a stub and coated with gold using a
209 JEOL JFC-1200 Sputter Coater. The samples were observed in a JEOL JSM-5200LV electron
210 microscope operating at 25 kV and with an analysis distance of 10/20 mm. Digital images were
211 directly captured.

212 Biofilm-forming ability was evaluated using the crystal violet (CV) and the ruthenium red (RR) assays
213 according to the procedures described by Lourenco et al. (2) and Borucki et al. (4), respectively, with
214 MWB as growth media.

215 Conformance to normality of the data generated by CV and RR assays was checked using the
216 Anderson-Darling test and conformance to homogeneity of variance was determined using Levene's
217 test. The comparison between wild type and mutant was then performed by ANOVA Least Significant
218 Differences (LSD) post hoc multiple comparison test by running the software Statistica® version 7.0
219 (Statsoft, Tulsa, OK, USA).

220

221 3. Results

222 Preliminary 2-D gel electrophoresis analysis of the exoproteome from planktonic and biofilm cells,
223 using a wide range pH gradient (3 - 10), showed that the majority of the proteins had their isoelectric
224 point (pI) in the acidic region (data not shown), therefore the analysis performed in this study used
225 IPG strips pH 4-7. Figure 1 shows representative gel images of three biological replicas for the two
226 strains in biofilm and planktonic form. The exoproteins from biofilms of strains *L. monocytogenes* 3119
227 and J311 are shown in Fig. 1A and Fig. 1C, respectively. The corresponding exoproteins from
228 planktonic state are shown in Fig. 1B and Fig. 1D. Approximately 472 spots were matched. The
229 protein spots that were significantly more intense in the biofilm state, for the joint analysis of the
230 strains, are numbered in the Figure 1 according to table 2.

231 For strain 3119, statistical analysis of the images indicated 40 spots (23 identified) with significant
232 higher intensity in biofilm state when compared with the planktonic state (average ratio > 1.5 and $p <$
233 0.05, Table 2). In the same analysis, 39 spots (29 identified) were significantly less intense in the
234 biofilm state. For strain J311, 32 spots (18 identified) had a significant higher intensity in the biofilm
235 state when compared with the planktonic state. Twenty eight spots (21 identified) were detected has
236 having significantly less intensity in the biofilm than in the planktonic state (Table 2).

237 Data from both strains was combined to strengthen the comparison between biofilm and planktonic
238 growth states. The joint analysis allowed the detection of 26 spots (16 identified) has having
239 significantly higher intensity in the biofilm state than in the planktonic state and 12 (10 identified) were
240 significantly more intense in the planktonic state.

241 The identification of these proteins indicated that PlcA, Lmo2504 and ActA were the proteins with
242 significantly higher intensity in the biofilm state compared with planktonic state, either for both strains
243 individually as for the joint analysis. On the other hand, for both strains individually and for the joint
244 analysis, antigen A and internalin C were among the proteins with the more negative ratios (Table 2).

245 With strain 3119, compared with strain J311, more spots, either with a positive or with a negative
246 ratio, were detected. Consequently, more differences between biofilm and planktonic state were
247 observed. In fact, when the spots with significant intensity and a positive ratio are compared, strain
248 3119 has 10 spots that were not considered on strain J311, whereas only five spots were not
249 significant for strain 3119. Likewise, on the negative ratio, strain 3119 has 13 spots that were not
250 considered on strain J311, whereas only five spots were not significant for strain 3119 (Table 2). It
251 can also be seen that, for strain 3119, the absolute values of the ratios are generally higher than for
252 strain J311, making strain 3119 a better candidate for the construction of a deletion mutant.

253 A deletion mutant on the gene coding for the protein with the second highest ratio between biofilm
254 and planktonic state (Lmo2504) was subsequently obtained on strain 3119. The resulting mutant
255 strain (3119 Δ Lmo2504) and the wild type strain were grown separately in triplicate in MWB at 25 °C
256 and growth rates (μ_{\max}) were for both strains approximately 0.072 h⁻¹.

257 The results obtained with crystal violet and ruthenium red assays for the mutant strain 3119 Δ Lmo2504
258 and its wild type are presented in Table 3. For both assays, either after 24 or 48 hours of growth, the
259 values obtained for the wild type strain were significantly higher than for the deletion mutant strain.
260 Figure 2 shows SEM images of 3119 and 3119 Δ Lmo2504. The wild type strain produced more
261 numerous and larger microcolonies as compared to the mutant.

262

263 4. Discussion

264 In this study some proteins were identified in more than one spot. This same effect has been verified
265 by other authors. Dumas *et al.* (15) stated that PlcB and InlC, from *L. monocytogenes* were present in
266 five and four spots, respectively, with different pIs and Mrs. These authors explained this effect by the
267 possible presence of protein orthologues with different Mrs, pIs, and/or posttranslational modifications
268 (isoforms). Trost *et al.* (16) on the analysis of extracellular and intracellular proteomes of *Listeria*
269 reported that following 2-DE, 120 spots were identified as belonging to 58 different proteins.

270 Posttranslational modifications or isoelectric focusing artefacts due to TCA precipitation or
271 desulphuration were pointed out by the authors as explanatory.

272 The major sources for protein isoforms include: variable spliced forms of the same gene product, (ii)
273 N- and C-terminal truncations, (iii) co- and post-translational modifications (includes modifications that
274 influence the charge of the protein such as phosphorylation, deamidation, glycosylation, and N-
275 terminal acetylation), (iv) endogenous protein degradation, and (v) oligomerisation (reviewed by Harry
276 *et al.*, 17). Protein modification by proteases that remove short peptides from either end of the
277 protein such as the cleavage and the removal of specific signal peptides will result in a unique
278 migration position on a 2-D gel. A narrow-range IPG allows the separation of isoforms that differ only
279 by a single amino acid substitution (reviewed by Harry *et al.*, 17). In this work, the narrow- range of
280 IPG (pH 4-7) used may also account for these results.

281 By comparing the exoproteomes of *L. monocytogenes* from biofilms and planktonic cells we have
282 detected and identified, for the two strains, 16 protein spots that were significantly more intense in the
283 biofilm state (Fig.1 and Table 2). Within this group of proteins were: phospholipase PlcA (Lmo0201),
284 flagellin (FlaA), a putative penicillin-binding protein (Lmo1438), an actin-assembly inducing protein
285 (ActA) and a putative cell wall binding protein (Lmo2504).

286 PlcA, phosphatidylinositol-specific phospholipase C is a *L. monocytogenes* virulence factor that
287 synergizes with listeriolysin O (LLO) and another phospholipase, PlcB, for the destabilization of the
288 membranes of primary and secondary phagosomes. PlcA, secreted by the Sec system, is positively
289 regulated by the positive regulatory factor A (PrfA) (18). Lemon *et al.* (19) have shown that this
290 virulence regulator has a significant role in biofilm formation. The transcription of *prfA* is under the
291 control of three promoters, including *PplcA* that is autoregulated by PrfA and results in a bicistronic
292 transcript for both *plcA* and *prfA*. Nevertheless, Lemon *et al.* (19) reported that a double mutant
293 lacking *plcA* and *plcB* unlike the $\Delta prfA$ mutant did not show defective biofilm formation.

294 Flagellin is a protein monomer secreted by the flagella export apparatus (FEA) that polymerizes to
295 form the flagella. The importance of flagella for biofilm establishment and development has been

296 reported (20, 21, 22). In a comparison of the whole proteome of *L. monocytogenes* cells from
297 planktonic and biofilm state, Tremoulet et al. (23) reported that flagellin was the only protein that
298 decreased in biofilm, compared to planktonic state. Nevertheless, Hefford et al. (24) observed higher
299 amounts of flagellin in biofilm grown cells than in planktonic cells. In the present study, the joint
300 analysis, with input from the two strains showed a significantly higher abundance of flagellin in the
301 biofilm exoproteome. However, when the comparison between growth states was performed
302 separately for each strain, only strain 3119 had a significantly ($p < 0.05$) and valid higher abundance
303 (average ratio > 1.5).

304 In both strains, a putative penicillin-binding protein (PBP) was observed at greater levels in the
305 exoproteomes of biofilm compared to the ones from planktonic state. The PBP's are a group of
306 proteins responsible for the final steps of peptidoglycan exoskeleton synthesis. In *L. monocytogenes*
307 EGDe 10 putative genes coding for PBP's have been identified (25). The protein identified in our
308 study is similar to the one coded by *lmo1438* of strain EGDe.

309 Ouyang et al. (22) verified the importance of a PBP for biofilm formation, as an insertion mutant of
310 this gene presented 86 % reduction in biofilm forming ability, compared to the wild type.

311 The actin-assembly inducing protein, ActA, is a virulence factor, as it allows assembly of actin from
312 the host cytoskeleton. The actin polymerization in one pole of the bacterial cell, forming the comet tail,
313 allows the propulsion of the bacteria towards the host cell's outer membrane (26). Travier et al. (27)
314 have pointed out a new role for ActA, showing the importance of PrfA and ActA for cell aggregation,
315 as the mutants for these genes displayed very low aggregation levels. Also, these authors found
316 evidence that the deletion of *actA* caused a reduction in biofilm formation, similar to the one caused
317 by the deletion of *prfA*. These authors further observed, by confocal microscopy, a thin and
318 homogenous layer biofilm for the *L. monocytogenes* $\Delta actA$ opposed to a mushroom-shaped and
319 dense biofilm from the wild type strain. The results presented here reinforce a possible extracellular
320 function of this protein as higher amount of ActA was detected in the biofilm exoproteomes from both
321 strains.

322 A predicted protein, coded by a gene homologous to *L. monocytogenes* EGD-e *lmo2504*, (*Lmo2504*)
323 was secreted by both strains in greater amounts in the biofilm state, especially for strain 3119 (Table
324 2). This protein, similar to cell wall binding proteins, is predicted to be secreted according to
325 Secretome 2.0 Server (28) with a signal peptide starting at position 1 and ending at position 25. Eight
326 conserved domains are identified for this protein, including a domain belonging to the peptidase
327 family M23, a group of zinc metallopeptidases (pfam 01551) (29). This group of enzymes may have
328 various functions involving murein hydrolytic activity, cell growth, cell wall turnover, peptidoglycan
329 maturation, cell division and separation, formation of flagella, sporulation, chemotaxis and biofilm
330 formation (30). An endopeptidase from this same family, along with other proteins with mureinic
331 activity from *Staphylococcus aureus*, has been shown to have influence on biofilm forming ability, as
332 the induction of a regulator for those genes increased their expression levels and an increase in
333 biofilm forming ability was verified (31).

334 *Lmo2504* may also have a role in a structural maintenance of chromosomes (SMC). Like *P45*,
335 *Lmo2504* has a SMC-related N-terminal domain and therefore may play a role in peptidoglycan
336 remodeling during segregation of the nucleoid (32).

337 Since *Lmo2504* was the second most abundant protein detected in the supernatants of both strains
338 when grown in biofilm state compared to planktonic state and since the most abundant one (*PlcA*)
339 had already been shown not to have influence on biofilm formation (19), a deletion mutant was
340 created on the gene coding for this protein and therefore access its influence in biofilm formation. The
341 deletion mutant strain *3119Δlmo2504* constructed in the present study allowed confirming the
342 importance of this protein in biofilm formation both by the crystal violet assay that mainly targets cells,
343 and by the ruthenium red assay that targets exopolysaccharides (Fig. 2). Observations using SEM
344 (Fig. 3) corroborate these results, as it was possible to observe larger and more numerous
345 microcolonies for the wild type strain and fewer and more dispersed for *3119Δlmo2504* indicating
346 lower attachment ability and a less structured biofilm of the mutant.

347 The role of proteins in biofilm development has been a subject of intense study in the recent years
348 and some progress has been made in several species. The role of biofilm associated proteins
349 (BAP's) has been established for Gram positive species such as *S. aureus*, *S. epidermidis* and
350 *Enterococcus faecalis*. These proteins present common characteristics in common such as high
351 molecular weight, the presence of tandem repeats, and the role in bacteria's infectious processes
352 (reviewed by Lasa and Penades 33). In the present work, the exoproteins more intensely detected in
353 the biofilm than in planktonic state are of lower molecular weight than those reported for BAPs.
354 Nevertheless, three of them are virulence factors (PlcA, ActA and InlC), two (Lmo2504 and Lmo0927)
355 have tandem repeats of small length and the protein ActA has a tandem repeat with potential
356 biological meaning according to T-REKS algorithm (34).
357 In this work we have explicitated differences in the exoproteomes of *L. monocytogenes* biofilms
358 versus its planktonic counterparts opening new avenues for future investigation. Furthermore, we
359 provide evidence for the importance of a cell wall binding protein in biofilm formation. These results
360 will assist in work to identify compounds capable of inhibiting the expression of proteins required for
361 biofilm production.

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371 References

- 372 1. **Mitjà, O., Pigrau, C., Ruiz, I., Vidal, X., Almirante, B., Planes, A. M., Molina, I., Rodríguez, D., Pahissa, A.**
 373 2009. Predictors of mortality and impact of aminoglycosides on outcome in listeriosis in a retrospective
 374 cohort study. *J. Antimicrob. Chemother.* **64**:416-423.
- 375 2. **Lourenço A, Rego F, Brito L, Frank JF.** 2012. Evaluation of methods to assess the biofilm-forming ability of
 376 *Listeria monocytogenes*. *J. Food Protect.* **75**:1411–1417.
- 377 3. **Karatan E, Watnick P.** 2009. Signals, regulatory networks, and materials that build and break bacterial
 378 biofilms. *Microbiol. Mol. Biol. Rev.* **73**:310–347.
- 379 4. **Borucki MK, Peppin JD, White D, Loge F, Call DR.** 2003. Variation in biofilm formation among strains of
 380 *Listeria monocytogenes*. *Appl. Environ. Microbiol.* **69**: 7336-7342.
- 381 5. **Longhi C, Scoarughi GL, Poggiali F, Cellini A, Carpentieri A, Seganti L, Pucci P, Amoresano A, Cocconcelli**
 382 **PS, Artini M, Costerton JW, Selan L.** 2008. Protease treatment affects both invasion ability and biofilm
 383 formation in *Listeria monocytogenes*. *Microb. Pathog.* **45**:45–52.
- 384 6. **Keller, M., Hettich, R.** 2009. Environmental proteomics: a paradigm shift in characterizing microbial
 385 activities at the molecular level. *Microbiol. Mol. Biol. Rev.* **73**:62-70.
 386 **Berrang ME, Meinersmann RJ, Frank**
 387 **JF, Smith DP, Genzlinger LL.** 2005. Distribution of *Listeria monocytogenes* subtypes within a poultry further
 processing plant. *J. Food Protect.* **68**:980-985.
- 388 7. **Leite P, Rodrigues R, Ferreira MASS, Ribeiro G, Jacquet C, Martin P, Brito L.** 2006. Comparative
 389 characterization of *Listeria monocytogenes* isolated from Portuguese farmhouse ewe's cheese and from
 390 humans. *Int. J. Food Microbiol.* **106**:111-121.
- 391 8. **Chakraborty, T., Leimeister-Wächter, M., Domann, E., Hartl, M., Goebel, W., Nichterlein T., Notermans,**
 392 **S.** 1992. Coordinate regulation of virulence genes in *Listeria monocytogenes* requires the product of the
 393 *prfA* gene. *J. Bacteriol.* **174**:568-574.

- 394 9. Premaratne RJ, Lin W, Johnson EA. 1991. Development of an improved chemical defined minimal medium
395 for *Listeria monocytogenes*. Appl. Environ. Microbiol. **57**:3046-3048.
- 396 10. Cabrita, P., Fonseca, C., Freitas, R., Carreira, R., Capelo, J. L., Trigo, M. J., Ferreira, R. B., Brito, L. 2010. A
397 secretome-based methodology may provide a better characterization of the virulence of *Listeria*
398 *monocytogenes*: preliminary results. Talanta. **83**:457-463.
- 399 11. Keller A, Nesvizhskii AI, Kolker E, Aebersold R. 2002. Empirical statistical model to estimate the accuracy
400 of peptide identifications made by MS/MS and database search. Anal Chem. **74**:5383-5392.
- 401 12. Nesvizhskii AI, Keller A, Kolker E, Aebersold R. 2003. A statistical model for identifying proteins by
402 tandem mass spectrometry. Anal. Chem. **75**:4646-58.
- 403 13. Arnaud M, Chastanet A, Débarbouillé M. 2004. New Vector for Efficient Allelic Replacement in Naturally
404 Nontransformable, Low-GC-Content, Gram-Positive Bacteria. Appl. Environ. Microbiol. **70**:6887-6891.
- 405 14. Dumas, E., Meunier, B., Berdagué, J.L., Chambon, C., Desvaux, M., Hébraud, M. 2008. Comparative
406 analysis of extracellular and intracellular proteomes of *Listeria monocytogenes* strains reveals a
407 correlation between protein expression and serovar. Appl. Environ. Microbiol. **74**:7399-7409.
- 408 15. Trost, M., Wehmhöner, D., Kärst, U., Dieterich, G., Wehland, J., Jänsch, L. 2005. Comparative proteome
409 analysis of secretory proteins from pathogenic and nonpathogenic *Listeria* species. *Proteomics*. **5**:1544-
410 1557.
- 411 16. Harry, J. L., Wilkins, M. R., Herbert, B.R., Packer, N. H., Gooley, A.A., Williams, K. L. 2000. Proteomics:
412 capacity versus utility. Electrophoresis. **21**:1071-1081.
- 413 17. de las Heras, A., Cain, R. J., Bielecka, M. K., Vázquez-Boland, J. A. 2011. Regulation of *Listeria* virulence:
414 PrfA master and commander. Curr. Opin. Microbiol. **14**:118-127.

- 415 18. **Lemon KP, Freitag NE, Kolter R.** 2010. The virulence regulator PrfA promotes biofilm formation by *Listeria*
416 *monocytogenes*. J. Bacteriol. **192**:3969–3976.
- 417 19. **Tresse O, Lebreton V, Garmyn D, Dussurget O.** 2009. The impact of growth history and flagellation on the
418 adhesion of various *Listeria monocytogenes* strains to polystyrene. Can. J. Microbiol. **55**:189–196.
- 419 20. **Lemon KP, Higgins DE, Kolter R.** 2007. Flagellar motility is critical for *Listeria monocytogenes* biofilm
420 formation. J. Bacteriol. **189**:4418–4424.
- 421 21. **Ouyang Y., Li J., Dong YQ., Blakely LV., Cao M.** 2012. Genome-wide screening of genes required for
422 *Listeria monocytogenes* biofilm formation. J. Biotechnol. Res. **4**:13–25.
- 423 22. **Trémoulet, F., O. Duché, A. Namane, B. Martinie., The European Listeria Genome Consortium, Labadie,**
424 **J. C.** 2002. Comparison of protein patterns of *Listeria monocytogenes* grown in biofilm or in planktonic
425 mode by proteomic analysis. FEMS Microbiol. Lett. **210**:25–31.
- 426 23. **Hefford MA, D'Aoust S, Cyr TD, Austin JW, Sanders G, Kheradpir E, Kalmokoff ML.** 2005. Proteomic and
427 microscopic analysis of biofilms formed by *Listeria monocytogenes* 568. Can. J. Microbiol., **51**:197–208.
- 428 24. **Korsak D, Markiewicz Z, Gutkind GO, Ayala JA.** 2010. Identification of the full set of *Listeria*
429 *monocytogenes* penicillin-binding proteins and characterization of PBPD2 (Lmo2812) BMC Microbiol.
430 **10**:239.
- 431 25. **Kocks C, Gouin E, Tabouret M, Berche P, Ohayon H, Cossart P.** 1992. *L. monocytogenes*-induced actin
432 assembly requires the actA gene product, a surface protein. Cell. **68**:521–531.
- 433 26. **Travier L, Guadagnini S, Gouin E, Dufour A, Chenal-Francisque V, Cossart P, Olivo-Marin JC, Ghigo JM,**
434 **Disson O, Lecuit M.** 2013. ActA Promotes *Listeria monocytogenes* Aggregation, Intestinal Colonization and
435 Carriage. PLoS Pathog. **9**:e1003131.

- 436 27. **Bendtsen JD, Kiemer L, Fausbøll A, Brunak S.** 2005. Non-classical protein secretion in bacteria. *BMC*
437 *Microbiol.* **5**:58.
- 438 28. **Finn RD, Mistry J, Schuster-Bockler B, Griffiths-Jones S, Hollich V, Lassmann T, Moxon S, Marshall M,**
439 **Khanna A, Durbin R, Eddy SR, Sonnhammer EL, Bateman A.** 2006. Pfam: clans, web tools and services.
440 *Nucleic Acids Res.* **34**:D247-D251.
- 441 29. **Popowska M.** 2004. Analysis of the peptidoglycan hydrolases of *Listeria monocytogenes*: multiple enzymes
442 with multiple functions. *Pol. J. Microbiol.* **53** Suppl:29-34.
- 443 30. **Dubrac S, Boneca IG, Poupel O, Msadek T.** 2007. New Insights into the Walk/WalR (YycG/YycF) Essential
444 Signal Transduction Pathway Reveal a Major Role in Controlling Cell Wall Metabolism and Biofilm
445 Formation in *Staphylococcus aureus*. *J. Bacteriol.* **189**:8257-8269.
- 446 31. **Bierne, H., Cossart, P.** (2007). *Listeria monocytogenes* surface proteins: from genome predictions to
447 function. *Microbiol. Mol. Biol. Rev.* **71**:377-397.
- 448 32. **Lasa I, Penades JR.** 2006. Bap: a family of surface proteins involved in biofilm formation. *Res. Microbiol.*
449 **157**:99-107.
- 450 33. **Jorda J, Kajava AV.** 2009. Identification of tandem repeats in sequences with a k-means based algorithm.
451 *Bioinformatics.* **25**:2632-2638.

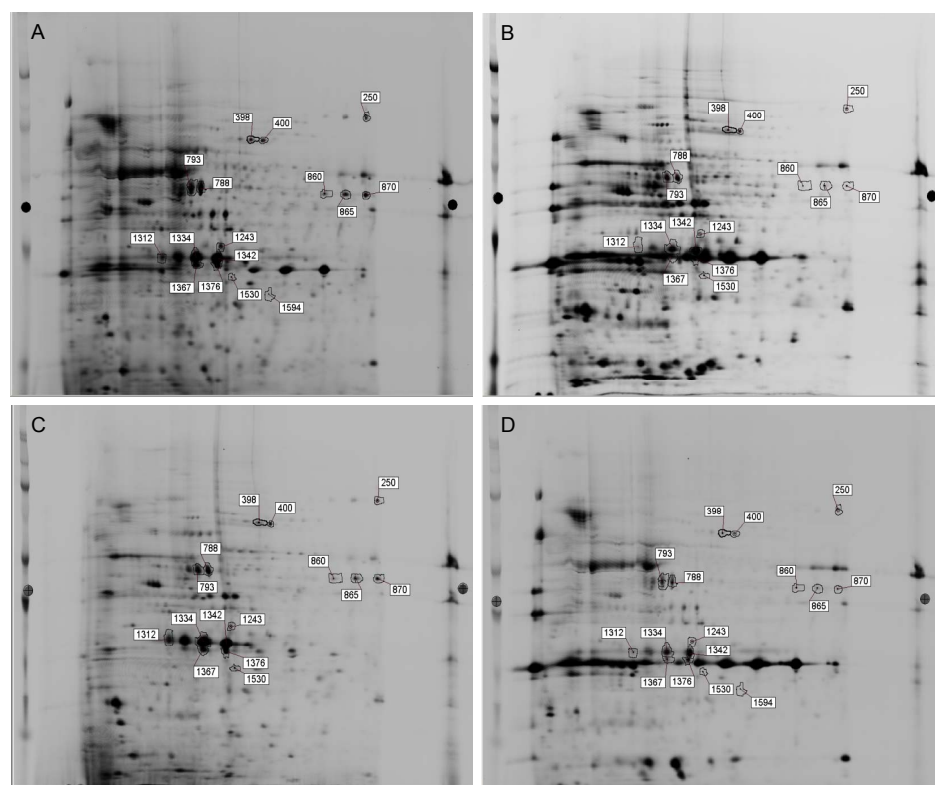


Figure 1- 2D-gel electrophoresis of the exoproteins of *L. monocytogenes* strains 3119 and J311 from biofilm (A and C) and from planktonic cells (B and D), respectively, on pH 3–7 IPG strips. Spots are numbered according to table 2.

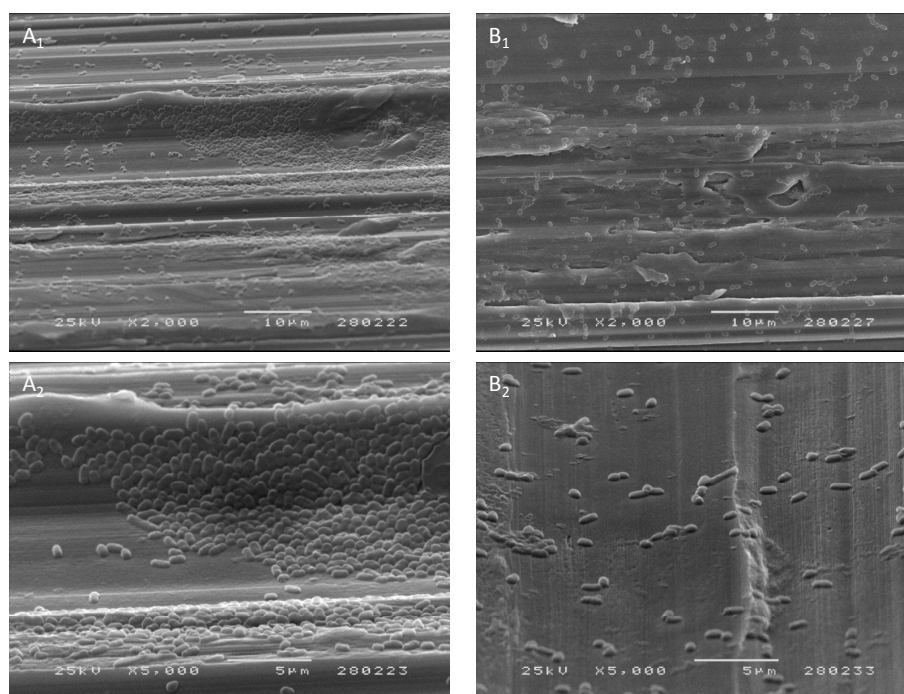


Figure 2 – Scanning electron microscope (SEM) images of strain 3119 (A₁ and A₂) and strain 3119Δmo2504 (B₁ and B₂) on stainless steel coupons. Biofilms grown in MWB for 48 h at 25 °C followed by media renewal and subsequent incubation of another 48 hours.

Table 1 – Primers used for construction and confirmation of strain 3119 Δ *mo2504*

Primer	5'	3'
P1	CGGGATCCACAGCTTTACGTGAA	
P2	TGGATCGACTGGAATTAGTGGCGCGGAAAT	
P3	ATTCCGCGCCACTAATTCCAGTCGATCCA	
P4	GGAATTCCACCCATTGCATCATA	
P5	CATGAATATGAAGCACAACA	
P6	ACCAACATCTCCATCGATTA	
P7	TGGTTATTTCCGCGCCACTA	
P8	GGTGCTGGATCGACTGGAAT	

Restriction sites for *Bam* HI and *Eco* RI are marked in bold in primers P1 and P4 sequences, respectively.

Table 2 – Exoproteins present in the supernatant fluids obtained from biofilms and planktonic cells, after six hours of secretion in MWB* at 25 °C.

Master Gel Spot Number	Biofilm/Planktonic						Identification **	Accession Number **	Gene **	unique pep	% cov.
	Joint analysis		3119		J311						
	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test					
1342	6.5	0.000026	9.54	0.0046	4.79	0.0047	PlcA	gil16802247	lmo0201	7	21
1334	4.55	0.000026	6.1	0.0046	3.56	0.0081	PlcA	gil16802247	lmo0201	6	18
250	4.53	0.001	7.17	0.0046	3.55	0.015	Lmo2504	gil16411992	lmo2504	5	19
870	4.03	0.0034	7.73	0.0046	2.98	0.045	Lmo2504	gil16411992	lmo2504	3	15
1243	3.34	0.000067	3.37	0.0074	3.31	0.013	actA actin-assembly inducing protein precursor	gil16802250	lmo0204	7	11
1312	3.3	0.00057	4.99	0.0055	-	-	flagellin	gil17433724	lmo0609	11	38
1376	2.75	0.00024	2.63	0.021	2.88	0.039	internalin C	gil16803826	lmo1786	11	39
865	2.63	0.014	4.94	0.0046	1.93	0.042	putative cell wall binding protein	gil225877514	lmo2504	5	19
400	2.39	0.00015	2.55	0.021	2.25	0.0073	putative penicillin-binding protein	gil16410867	lmo1438	4	25
1594	2.24	0.0003	2.76	0.0053	-	-	upp	gil16412026	lmo2530	6	34
788	2.17	0.0014	3	0.0095	1.61	0.037	hypothetical protein Lmo0927	gil16802967	lmo0297	3	6.9
1367	2.11	0.000048	2	0.0081	2.25	0.0047	internalin C	gil16803826	lmo1786	14	49
1530	1.74	0.042	2.85	0.014	-	-	indole-3-glycerol phosphate synthase	gil21363054	lmo1630	7	27
398	1.7	0.002	1.87	0.044	1.56	0.0094	putative penicillin-binding protein	gil16410867	lmo1438	20	36
860	1.7	0.05	2.85	0.0076	-	-	Lmo2504	gil16411992	lmo2504	2	11
793	1.53	0.023	2.21	0.0091	-	-	hypothetical protein Lmo0927	gil16802967	lmo0927	9	16
397	-	-	1.55	0.026	-	-	putative penicillin-binding protein	gil16410867	lmo1438	6	12
773	-	-	-	-	1.84	0.02	glucose-6-phosphate isomerase	gil16804405	lmo2367	9	22
936	-	-	-	-	1.71	0.0073	enolase	gil16411943	lmo2455	18	49
1000	-	-	2.31	0.014	-	-	peptidoglycan lytic protein P45	gil16804543	lmo2505	5	19
1023	-	-	-	-	1.64	0.028	glyceraldehyde-3-phosphate dehydrogenase	gil16804497	lmo2459	12	52
1134	-	-	-1.77	0.0063	-	-	translation elongation factor Ts	gil16803697	lmo1657	6	22
1255	-	-	2.71	0.017	-	-	6-phosphofructokinase	gil16803611	lmo1571	8	29
1283	-	-	2.08	0.03	-	-	putative AA3-600 quinol oxidase subunit II	gil16802061	lmo0013	3	12
1414	-	-	-	-	-6.76	0.028	internalin C	gil16803826	lmo1786	5	24
1425	-	-	-	-	-6.65	0.014	internalin C	gil16803826	lmo1786	10	32

Table 2 – (continued)

Master Gel Spot Number	Biofilm/Planktonic						Identification **	Accession Number **	Gene **	unique pep	% cov.
	Joint analysis		3119		J311						
	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test					
1433	-	-	-3.97	0.013	-11.22	0.0047	internalin C	gil16803826	lmo1786	15	64
1436	-	-	-2.63	0.016	-7.55	0.014	internalin C	gil16803826	lmo1786	15	63
1615	-	-	-2.45	0.013	-	-	hypothetical protein Lmo0125	gil16802173	lmo0125	10	53
1618	-	-	-2.37	0.0065	-	-	hypothetical protein Lmo0125	gil16802173	lmo0125	11	51
1702	-	-	-1.54	0.014	-	-	Antigen A	gil16802166	lmo0118	5	34
1736	-	-	-1.98	0.013	-	-	Antigen A	gil16802166	lmo0118	5	27
1800	-	-	4.56	0.0064	-	-	Antigen A	gil16802166	lmo0118	4	30
1886	-	-	-2.13	0.018	-	-	antigen B	gil16802165	lmo0117	4	26
1894	-	-	7.6	0.0046	2.87	0.016	listeriolysin O precursor	gil16802248	lmo0202	8	15
1962	-	-	-6.1	0.046	-	-	actA actin-assembly inducing protein precursor	gil16802250	lmo0204	4	6.6
1987	-	-	-2.01	0.014	-4.97	0.0085	listeriolysin O precursor	gil16802248	lmo0202	2	4.5
2007	-	-	-2.09	0.014	-10.4	0.0077	P60 extracellular protein	gil1171970	lmo0582	4	9.9
2015	-	-	-8.46	0.0055	-	-	listeriolysin O precursor	gil16802248	lmo0202	2	4.5
2034	-	-	-9.02	0.0046	-	-	listeriolysin O precursor	gil16802248	lmo0202	2	4.5
2058	-	-	-9.29	0.0053	-	-	30S ribosomal protein S10	gil16804671	lmo2633	2	23
2097	-	-	-2.1	0.0065	-	-	listeriolysin O precursor	gil16802248	lmo0202	2	4.5
2118	-	-	-	-	-1.95	0.034	listeriolysin O precursor	gil16802248	lmo0202	2	4.5
2276	-	-	-9.65	0.014	-6.58	0.034	internalin C	gil16803826	lmo1786	14	64
2279	-	-	-	-	-2.1	0.016	listeriolysin O precursor	gil16802248	lmo0202	15	38
2280	-	-	-2.49	0.0074	-	-	Lmo0127	gil16409486	lmo0127	10	58
2281	-	-	-	-	-3.91	0.0077	P60 extracellular protein	gil1171970	lmo0582	4	11
2282	-	-	-	-	1.93	0.014	glyceraldehyde-3-phosphate dehydrogenase	gil16804497	lmo2459	10	42
2283	-	-	-	-	2.9	0.021	listeriolysin O precursor	gil16802248	lmo0202	2	4.5
2284	-	-	-12.35	0.0091	-11.01	0.013	internalin C	gil16803826	lmo1786	18	53
2287	-	-	-9.43	0.0053	-9.99	0.0047	internalin C	gil16803826	lmo1786	16	47

Table 2 – (continued)

Master Gel Spot Number	Biofilm/Planktonic						Identification **	Accession Number **	Gene **	unique pep	% cov.
	Joint analysis		3119		J311						
	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test					
2288	-	-	6.22	0.0063	2.8	0.038	Lmo2504	gil16411992	lmo2504	3	8.9
685	-2.2	0.04	-1.75	0.03	-2.45	0.0047	listeriolysin O precursor	gil16802248	lmo0202	4	10
678	-2.44	0.018	-1.91	0.012	-2.78	0.0077	listeriolysin O precursor	gil16802248	lmo0202	16	33
1379	-6.26	0.00018	-6.23	0.011	-6.32	0.013	internalin C	gil16803826	lmo1786	15	56
878	-7.68	0.00018	-7.76	0.0064	-7.63	0.0047	actA actin-assembly inducing protein precursor	gil16802250	lmo0204	8	13
1078	-8.14	0.00037	-8.81	0.0063	-7.84	0.0055	actA actin-assembly inducing protein precursor	gil16802250	lmo0204	15	26
1510	-8.27	0.0000068	-7.12	0.0053	-9.27	0.0055	Lmo0129	gil16409488	lmo0129	9	41
1712	-9.32	0.004	-5.25	0.019	-11.42	0.014	antigen A	gil16802166	lmo0118	3	20
1410	-11.52	0.0044	-14.91	0.013	-10.78	0.0047	internalin C	gil16803826	lmo1786	4	18
1435	-13.54	0.0003	-15.32	0.0055	-12.89	0.0062	actA actin-assembly inducing protein precursor	gil16802250	lmo0204	11	18
1382	-14.34	0.00017	-16.95	0.0074	-	-	internalin C	gil16803826	lmo1786	2	12

Positive ratio values indicate higher abundance in the biofilm state. Negative ratios indicate higher abundance in the planktonic state.

The spots ratios marked with a dash did not comply with the established cutoff parameters for considering a valid difference of protein abundance between biofilm and planktonic states (absolute value of ratio > 1.5 with p < 0.05).

*MWB – Modified Welshimer's broth

** According to EGDe strain

Table 3 - Biofilm forming ability of strains 3119 and 3119 Δ *Imo2504* using the crystal violet assay (Abs_{600nm}) and the ruthenium red assay (Abs_{450nm}) after growth for 24 or 48 h in MWB at 25 °C.

Strain	Crystal violet		Ruthenium red	
	24 hours	48 hours	24 hours	48 hours
3119	0.1412 \pm 0.0044	0.2561 \pm 0.0164	0.3546 \pm 0.0865	0.3440 \pm 0.0134
3119Δ<i>Imo2504</i>	0.1331 \pm 0.0048	0.2101 \pm 0.0096	0.1320 \pm 0.0527	0.2105 \pm 0.0774
Comparison p value*	0.0158	0.0064	0.0041	0.0149

Average values \pm standard deviation are indicated.

*p values obtained using ANOVA *post hoc* LSD comparison test. For both assays and both times, the level of significance considered was $p < 0.05$ and therefore differences between wild type and mutant were significant.